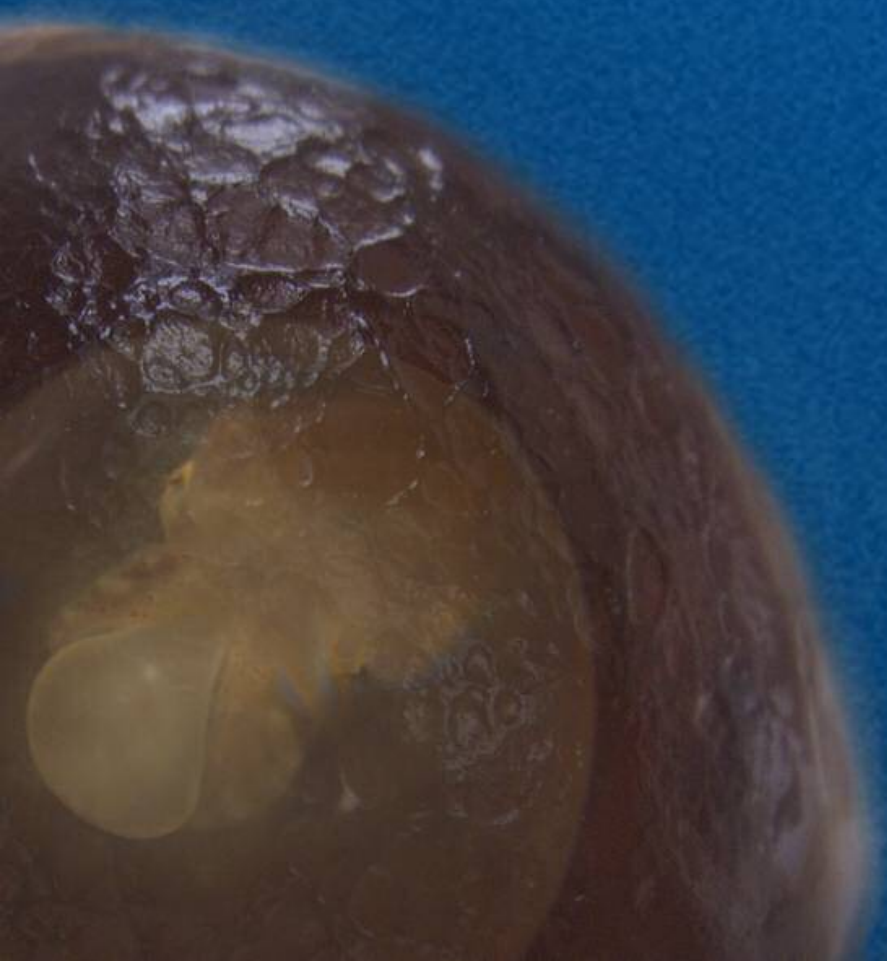
Invertebrate predation on egg masses of the European cuttlefish, *Sepia officinalis*: an experimental approach

Catarina Padilha Pires Martins



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ABSTRACT

Sepia officinalis embryos develop inside ink-stained black encapsulated eggs fixed to the seafloor in shallow water habitats. The enveloping layers of these eggs allow the embryo to develop within a micro-environment and are thought to provide protection. Since embryological development may last months, this short-lived species spends a relatively long period of its lifespan on shallow waters, vulnerable to predation and physical stress. Yet, only a handful of species are known to feed on *S. officinalis* eggs, most of them fish. Marine benthic invertebrates, however, are likely candidates contributing to predation of this critical life stage. In this study, several invertebrate marine species, from 6 different phyla and with diverse feeding habits, were investigated as potential predators of *S. officinalis* eggs under laboratory conditions. Feeding experiments also tested to what extent the egg capsule and lower levels of mechanical protection could deter predation and Multivariate Correspondence Analyses were performed to explore which feeding traits of the tested invertebrate species would explain the experimental results. Results showed predation on eggs by crabs and echinoderms equipped with certain prey capture methods that allowed them to feed on eggs. Also, the egg capsule appeared to provide protection from predation by certain species with less powerful prey capture tools. Prey capture tools of the tested species appeared to be the feeding trait that better explained the experimental results. Therefore, whether or not eggs were eaten in these experiments might be determined by a mechanical factor. Nonetheless this may not be the case for species that might not have fed on eggs due probably to a chemosensory question, not recognising them as food. This work thus contributes to the understanding of the ecology of early life stages of cuttlefish and the factors that can affect offspring survival and subsequently impact recruitment to the adult populations of this exploited species.

ACKNOWLEDGMENTS

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1. INTRODUCTION

Predation is an important source of egg mortality for many animals (Orians & Janzen 1974) and encapsulation of eggs is a common protection strategy among marine invertebrates (Roche et al. 2011). While some animals appear to be specialists at eating eggs, almost all carnivores and many omnivores include several kinds of eggs in their diets (Orians & Janzen 1974). For instance, the nemertean *Carcinonemertes regicides* is a well-documented egg predator (Kuris et al. 1991) and certain echinoderms and crabs feed on gastropod eggs (Bigatti et al. 2010, Dumont et al. 2008, Perron 1981). Generally, large eggs with longer developmental periods require more protection than small, more quickly-developing ones (Perron 1981). Therefore, egg capsule walls with strong structures, which constitute an energetic cost, may have evolved as a result of selection for improved protection of embryos from predation and physical stress during a protracted period of development (Perron 1981). Additionally, in coastal environments, egg capsules also protect embryos from various environmental stresses, bacterial attack and wave action (Roche et al. 2011).

Cephalopods display a diversity of egg morphologies, structure and size and in most cases the eggs are relatively well protected by encapsulation (Boletzky 2003). Cephalopod embryos are generally not viable if they are not properly protected and primary protection is provided by the chorion membrane, which is considered insufficient by itself (Boletzky 2010). Therefore, protection might be ensured through other means. In benthic and bentho-pelagic cephalopods, this protection is provided mainly in two ways: hard egg capsules that envelope eggs that develop without maternal care or egg brooding, a form of active protection in which the female stays with the eggs during their entire development while also ensuring that the conditions are favourable (Boletzky 1986). In fact, cephalopods with brooding include only a few oegopsid squid species and all incirrate octopods. Incirrate octopods are the only cephalopod group that does not have additional protective structures other than the chorion membrane, whereas all other cephalopods appear to have eggs also enwrapped and protected by capsules or jelly masses, which constitute the most important barrier against environmental damages (Boletzky 1998).

1.1. European cuttlefish *Sepia officinalis*

Cuttlefish are among the most important commercial cephalopod resource in European waters and although several species are present in the north-east Atlantic, landings of the European cuttlefish *Sepia officinalis* dominate (Bloor et al. 2013). Additionally, *S. officinalis* is used as an animal model for biological and biomedical research, is recognised as a species with great potential for aquaculture and is used for public exhibition in aquariums (Sykes et al. 2014). Its distribution extends through the Mediterranean Sea and the waters of the north-eastern Atlantic to the north-western coast of Africa (Bloor et al. 2013). It is a nektobenthic species that lives in habitats with sandy, muddy and rocky substrate from the coastline (2 – 3 m depth) to depths of up to 200 m (Bloor et al. 2013, Guerra 2006). It belongs to one of the few cephalopod groups with a chambered shell, known as cuttlebone in *Sepia*, a rigid calcium carbonate structure responsible for buoyancy control that also limits its depth range (Sherrard 2000).

1.1.1. Reproduction and egg characteristics

Sepia officinalis generally has an annual lifespan, ranging from 12 to 18 months (Guerra 2006) and is categorised as an intermittent spawner, having a single spawning period that occurs at the end of its life cycle (Rocha et al. 2001). Spawning is temperature dependent, occurs in shallow coastal waters between 5 and 60 m depth (Guerra et al. 2016) and its duration may vary over the geographical range of the species (Bloor et al. 2013).

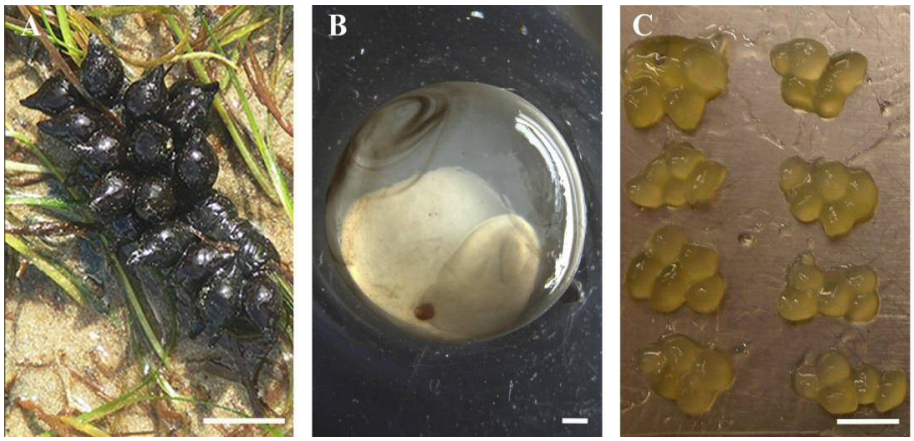

Fig. 1. *Sepia officinalis*. (A) Eggs in their natural condition (with egg capsule). (B) Egg without egg capsule; embryo is surrounded by the chorion membrane. (C) Mature oocytes. A, C scale bars: 1 cm. B scale bar: 1 mm.

S*epia officinalis* lays eggs individually in clusters attached to a variety of fixed natural substrates (Fig. 1A) such as plants, algae, sessile animals, moving animals and previously deposited cuttlefish eggs, or even, when available, artificial substrates, typically fishing traps or nets (Bloor et al. 2013, Lacoue-Labarthe et al. 2016).

The process of spawning (illustrated in Fig. A1A) starts with the maturation of oocytes, cells ~7 mm in size (Laptikhovsky et al. 2003), in the female ovary, where follicular cells secrete the chorion membrane, which surrounds mature oocytes and constitutes the first envelope of the egg (Boletzky 1986). These oocytes are then released through the oviduct and, after mating occurs, are transported by contractions of the distal oviduct and released individually into the mantle cavity (Zatylny et al. 2000). Here, each mature oocyte receives a first gelatinous envelope secreted by the oviducal glands, followed by a second gelatinous envelope composed of several layers secreted by the nidamental glands and stained with ink released by the ink sack (Boletzky 1986, Cornet et al. 2015, Zatylny et al. 2000). These envelopes constitute what is known as the egg capsule. The enveloped oocytes then proceed into the funnel tube and are brought to the base of the ventral arms, close to the mouth (Zatylny et al. 2000). Spermatozoids, which are stored in special structures under the buccal mass of the female, are then released and fertilization occurs (Zatylny et al. 2002). To fertilize the oocyte, the spermatozoid crosses the freshly produced jelly envelopes that surround the oocyte, which are soft and gelatinous prior to fertilization (Boletzky 1986, Zatylny et al. 2002). Following fertilization, the perivitelline fluid (PVF) is formed and numerous changes are triggered in these envelopes, which allow the creation of a micro-environment for embryological development (Boletzky 2003, Cronin & Seymour 2000). The embryo has an embryological life that can be divided into 30 stages (Boletzky et al. 2016) and develops bathed in the PVF and surrounded by the chorion membrane and the egg capsule (Fig. A1B), which provide physical and chemical protection against the surrounding environment (Cornet et al. 2015, Lacoue-Labarthe et al. 2016). Nevertheless, the encapsulation of embryos within these protective layers comprises a considerable energetic investment by the female (Boletzky 2003).

1.1.2. Egg capsule characteristics

To deposit each egg, the female uses the arm tips to pull the gelatinous egg envelope secreted by the nidamental glands into two filaments and wraps them around the

substrate, forming a ring that fixates the egg (Boletzky 1983, Boletzky 1986) (Fig. 1). Freshly laid eggs are soft and gelatinous (Boletzky 1983) and the egg capsule quickly hardens and becomes thicker (Lacoue-Labarthe et al. 2016). The initial thickness is approximately 1.5 mm and then decreases throughout embryonic development, while still retaining elasticity to allow for embryonic growth (Cornet et al. 2015, Wolf et al. 1985). Although the egg capsule allows for oxygen diffusion throughout embryological development, it acts as a shielding barrier for most of this period hindering the penetration of contaminants and most metallic ions within the egg (Lacoue-Labarthe et al. 2016). However, during the last third of embryological development, coinciding with the highest embryonic oxygen demands, this structure becomes more permeable due to the expansion of the egg capsule (Fig. A2D), caused by the embryo release of solutes into the PVF that create an osmotic pressure and provoke a substantial volume increase of the PVF (Cronin & Seymour 2000).

The ultrastructure of the egg capsule reveals an organised network of glycoproteins and polysaccharides with elasticity and resistance properties (Cornet et al. 2015). Within this network, melanin deposits and symbiotic bacteria are also found (Cornet et al., 2015). Melanin is one of the main components of cephalopod ink, comprising 15 % of its total wet weight (Derby 2014). Cephalopod ink has been proposed as an aversive deterrent of predators or a disruptor of chemical senses of predators and is thought to defend cephalopods against them in a variety of ways (Derby 2014). For instance, it has been proposed as phagomimetic defence due to its high levels of amino acids, which are strong phagostimulants of marine predators (Derby 2014). On the other hand, ink from two squid species has been shown to be unpalatable to predatory fish (Derby 2014) and it has been speculated that quinones, which are often used as chemical defenses by animals, might constitute the feeding deterrents in *Sepia* ink (Prota et al. 1981). Furthermore, the mentioned symbiotic bacteria populations lodged within the egg capsule, found also in eggs of other cephalopod species, are thought to provide protection throughout the embryological development by inhibiting colonization of other bacteria or actively producing antimicrobial substances (Biggs & Eppel 1991, Cornet et al. 2015). In addition, egg capsule proteins also appear to have antimicrobial and antifouling properties (Cornet et al. 2015). Nevertheless, it should be noted that the chemical composition of the egg capsule is still largely unknown (Boletzky et al. 2016).

1.1.3. Embryological stage and egg predation

Sepia officinalis eggs, measuring ~20 mm length and ~10 mm diameter (Boletzky 1983), are among the largest cephalopod eggs (Boletzky 1983) and because these are attached to structures on the seabed, they stay on the same space a significant period of time. In result, their development is dependent on the surrounding spawning ground conditions and so, the spawning site can affect dramatically the survival and fitness of offspring (Bloor et al. 2013). However, the process by which sexually mature *Sepia* females select their spawning habitat is unknown (Bloor et al. 2013).

Duration of egg development varies with water temperature, ranging from approximately one month at 25 ºC to three months at 15 ºC (Boletzky 1983, Lemaire 1970). For this short-lived species, this constitutes a relatively long period of its lifespan (Lacoue-Labarthe et al. 2016). No parental care has been reported in this species and yet no major predation pressure on the eggs has been reported either (Guerra 2006). Nonetheless, a limited number of predation events on *Sepia* eggs in the wild have been documented. On *S. officinalis* eggs, predation has been observed in the Ria de Vigo (north-eastern Atlantic) by the Tomblot blenny *Parablennius gattorugine* (Guerra & González 2011), in Morbihan Bay (north-eastern Atlantic) by the triggerfish *Balistes capriscus* (Blanc & Daguzan 1999) and in the north-western Adriatic Sea by the gastropod *Hexaplex trunculus* (Melli et al. 2014). On *Sepia pharaonis* eggs, predation has been observed in the Arabian Sea by the fish families Chaetodontidae, Balistidae, Monacanthidae and Zanclidae (Gutsal 1989). On *Sepia latimanus* eggs, predation has been observed in Guam (south Pacific) by the butterfly fish *Chaetodon ulietensis* (Corner & Moore 1980). The few works that look at egg predation is noticeable but it is also interesting to note the almost complete absence of invertebrates from these accounts. Although a rich diversity of benthic invertebrates are bound to encounter the conspicuous siting targets that are the black masses of *Sepia* eggs, only one invertebrate species is mentioned.

Interestingly, other *Sepia* species with unstained egg capsules do show behaviours during spawning that provide complementary egg protection against predators. For instance, *Sepia orbignyana* females insert eggs into the oscula of sponges, which provide camouflage (Boletkzy 1986), *Sepia elegans* females fix their eggs on braches of octocorallians so polyps surrounding the egg may protect it (Boletzky 1986) and *S.*

latimanus places its eggs in crevices (Corner & Moore 1980). Furthermore, during this embryological stage, development and growth of the embryo is fuelled by energetic reserves stored as yolk, a nutritious structure rich in proteins, carbohydrates and lipids (Matozzo et al. 2015) and of which *S. officinalis* embryos are provisioned generously (Boletzky 2003). Therefore, eggs would be rather nutritious targets for predators.

1.1.4. Hatching and the process of breaking through the physical barriers

Since *S. officinalis* embryos develop within this physical barrier composed by the chorion membrane and the egg capsule, they have strategies to penetrate and hatch through these relatively thick envelopes at the end of embryonic development. This is accomplished mainly by the Hoyle organ; a hatching gland located on the posterior tip of the mantle that releases enzymes that digest the chorion membrane and the egg capsule, combined with muscular contractions of the mantle (Cyran et al. 2013). In *Sepia*, the Hoyle organ consists of thin bands of glandular cells with globular secretory granules that are released individually by exocytosis and densely ciliated non-secretory cells (Cyran et al. 2013). However, which types of enzymes are involved in the digestion of the chorion and the capsule remains unclear (Cyran et al. 2013). Also, egg capsule proteins might be cleaved to allow hatching (Cornet et al. 2015).

2. OBJECTIVES

Experiments were set up to investigate potential natural predators of the cuttlefish *Sepia officinalis* eggs and explore to what extent the egg capsule and lower levels of mechanical protection can deter their predation.

3. MATERIALS AND METHODS

3.1. Collection of animals

Cuttlefish (*Sepia officinalis*) eggs were obtained from egg masses spawned in the laboratory by captive adults collected from the artisanal fishery of Vilanova i la Geltrú, NW Mediterranean, from February to April of 2016, and maintained at the Zona de Acuarios Experimentales (ZAE) of the Institut de Ciències del Mar (ICM). Additional egg masses spawned in aquaria were also provided by the public aquarium of Barcelona (*l'Aquàrium*). During the experimental period, egg masses were incubated at a temperature of 14 ± 1 ºC, (ranging from 12 – 17 ºC). Egg mases were incubated

attached to the structure used by the female to spawn, usually plastic ropes; isolated eggs were incubated inside laboratory tanks (see below) using plastic, perforated floating containers.

Potential predator specimens were obtained from different sources: local trawler fishery of Blanes and Vilanova i la Geltrú (NW Mediterranean), fishery and bait markets, collected directly from nature (Muros de Nalón, NE Atlantic) and some of them were also provided by *l'Aquàrium*. A total of 22 invertebrate species, representing 6 different animal phyla (Cnidaria, Nemertea, Mollusca, Annelida, Arthropoda and Echinodermata), were tested as potential predators (Fig. A3). The number of the animals tested per species, mean fresh body size and weight, origin and collection method of specimens is presented in Table A1. Potential predators were selected to represent a wide taxonomic range with different feeding diets and habits and that could come across egg masses of *S. officinalis* in their natural habitat (Table A2). Bathymetric and feeding characterization of these species is summarized in Table A2.

3.2. Laboratory experiments (Fig. 2A)

Experiments were conducted at the ZAE from April 26 to August 3, 2016. Potential predators were placed in chambered cages (47 x 45 x 26 cm) of green wire mesh of 10 x 10 mm (Fig. A4A), preventing the escape of animals and ensuring adequate water circulation, inside 120 l tanks (116 x 71 x 21 cm) supplied with running fresh seawater (5.5 $l \cdot min^{-1}$). Cages were opened from the top and had the bottom reinforced with a green plastic mesh of 1 x 1 mm. During the experiments, a dark net was placed on top of the experimental cages to avoid potential escapes and minimize light disturbances. Cages and tanks were both cleaned before each experiment. All animals were kept under natural photoperiod and water temperature was 16 ± 1 ºC (ranging from 13 to 18 ºC).

3.2.1. Experimental design and procedure

Potential predators were placed individually in the chambers (23 x 14 x 25 cm) and animals bigger than the experimental chambers were placed instead in baskets (31 x 23 x 20 cm) (Fig. A4B), where the experiments were performed following the same methodology. Feeding experiments consisted of presenting potential predators with three food options (Fig. 1): (a) 5 cuttlefish eggs with capsule (EC), (b) 5 cuttlefish eggs

without capsule (EWC) and (c) 5 cuttlefish mature oocytes. These food options have a similar biochemical composition and represent decreasing degrees of mechanical protection, due to the different properties of its outer physical barrier; solid, turgid and flaccid correspondingly. EC are the natural condition of *S. officinalis* eggs and have a solid physical barrier (the egg capsule), which represents the highest degree of protection in this experimental design. EWC are protected by the expanded chorion membrane onset by fertilization and the emergence of the perivitelline fluid and its subsequent incorporation of water (Boletzky 2003). At this stage, the chorion membrane constitutes a turgid physical barrier due to the tensional strain created by the perivitelline fluid, which in turn provides and maintains the rigidity of the membrane. To obtain EWC, the egg capsule was removed by hand to its maximum extent, taking care not to rupture the chorion membrane or weaken it so much that it might rupture spontaneously. Since it was logistically impossible to present all test animals with eggs of identical developmental stage, a developmental stage range was used. Following the embryological developmental stages proposed by Boletzky et al. (2016), potential predators were presented with eggs at stages 21-28 (Fig. A2A-C). OO are cells whose outer layer is the chorion membrane prior to its expansion and the changes caused by fertilization and embryological development. Therefore, OO are surrounded by a flaccid physical barrier, which represents the lowest level of protection in this experimental design. Unlike EC and EWC, OO are not a balloon-like structure and from a mechanical perspective, are also easier to manipulate underwater. OO were obtained from the ovary of a dissected *S. officinalis* mature female.

Potential predator individuals were presented with either of the three food options (EC, EWC, or OO) and were used only once. Prior to running the experiments, potential predators were starved for at least 48h. In the case of a few of the animals, their condition was assessed prior to the experiments by offering a small piece of mantle tissue of mussel. Having eaten the offered piece of mussel, these individuals were then placed in starvation before starting the experiment. Animals that did not eat the food option of the experiment were assessed visually or by offering some other piece of food more attractive to the animal after the experiments. Experiments lasted for 48h or until all food items had been eaten. Animals were observed throughout the experiments during daytime hours and a few predation events were recorded. In the case of

Astropecten aranciacus, feeding of EC was purposefully recorded in a glass aquarium (28 x 22 x 15 cm) with running fresh seawater.

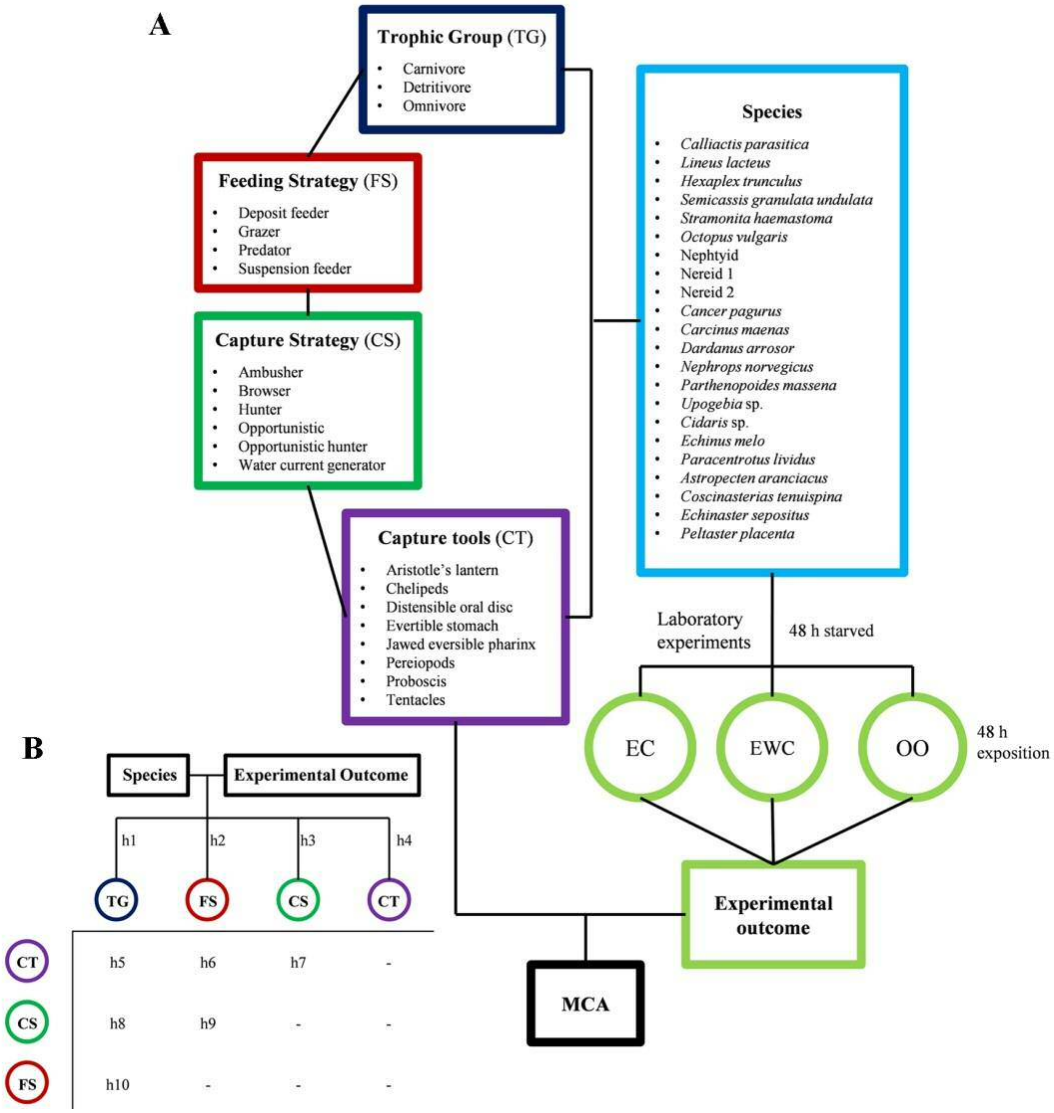

Fig. 2. Diagrams of the study. (A) Diagram outlining the global structure of the study. Square box in light blue indicates the species tested as potential predators of *Sepia officinalis* eggs in laboratory. Circular boxes represent the laboratory experiments and squared boxes represent the variables used in the MCA analyses. Squared boxes colour coded represent the four biological categories (and its corresponding levels) used to characterize the feeding biology of the tested potential predator species. (B) Diagram outlining the construction of all 10 hypotheses from combinations of variables to perform the MCA analyses. Black boxes represent the 2 variables used in all analyses. Circular boxes represent the other 4 biological variables (colour coded) considered as potentially explanatory of the experimental results and that differed between hypotheses. (CS, capture strategy; CT, capture tools; EC, egg with capsule; EWC egg without capsule; FS, feeding strategy; h1, hypothesis 1; h2, hypothesis 2; hypothesis 3; and so on; MCA, Multivariate Correspondence Analysis; OO, oocytes; TG, trophic group).

At the end of each experiment, the number of eggs was counted and the potential predator subjects were measured (see Table A3 for the definition of the body size measurement used to characterize each species) and weighed. EC with visible marks on the surface of the capsule were examined under binocular microscope and when EC were ruptured, these were opened to check whether the embryo was still inside. Video recordings of the predatory behaviour were obtained using a video camera SAVFY SJ4000 1080P. Although 22 potential predator species were tested in total, only 16 of these were presented with all 3 options of the feeding experiment due to insufficient individuals available to perform all trials. This meant that, for example, none of the molluscan species was tested against the complete feeding experiment. A few modifications were made to the standard methodology to adapt it to the biological diversity of our experimental potential predatory subjects, in particular for the species *Calliactis parasitica*, *Lineus lacteus*, *Octopus vulgaris*, the nephtyid and nereid polychaetes and *Upogebia* sp.

Anemone *Calliactis parasitica*

This anemone was selected as a potential predator due to a previous observation of a predatory behaviour on *S. officinalis* eggs during its mutualistic association with the crab *Dardanus arrosor.* Individuals of *C. parasitica* were carefully removed from the shell of *D. arrosor* individuals and placed each in a glass Petri dish (14 cm diameter). The Petri dishes were then placed inside the experimental chambers, where the experiments were performed. In this case, we waited until individuals had the oral disc open and then offered to the anemone 1 EC, 1 EWC or 1 OO depending on the experiment, by dropping it gently by hand over the oral disc. We then observed the behaviour of the animals and their initial reaction to the food items was video recorded. Experiments were considered complete when the anemone consumed the offered item and for those that did not do so on first contact, experiments lasted for 48 h during which the egg offering operation was repeated 7-10 times.

Nemertean *Lineus lacteus* and the nereid polychaetes

Due to the small size and admirable escape behaviour of the nemertean *L. lacteus* and nereid polychaetes, the experiments with these animals were performed in closed 0.6 l plastic containers (Fig. A4C) filled with fresh seawater, which was renewed daily. The

experiments were then conducted in the same manner as those described in the standard methodology.

Common octopus *Octopus vulgaris*

The feeding experiment performed with *O. vulgaris* followed the same design as the standard methodology described above, but took place in a 2500 l circular tank with running fresh seawater, where the individual had been maintained and was fed daily live *Carcinus maenas* crabs. Before initiating the experiment, the tank was cleaned and the octopus was starved for 48 h. After the presentation of the EC, the animal was recorded and it was then checked on periodically throughout the experiment.

Nephtyid polychaete and the mud lobsters *Upogebia* sp.

Because the nephtyid polychaetes and the mud lobster *Upogebia* sp. are small in size (Table A1), the designed chambered cages of the standard methodology were useless to hold the animals and prevent their escape. These were placed in 0.6 l plastic containers (Fig. A4C) with round openings covered with a 500 µm net and were submerged in the tanks used in the standard methodology. This design allowed a continuous flow of water while also preventing the escape of the animals. Otherwise, the experiments were performed following the standard methodology.

3.1. Statistical analyses

Chi-square analyses on the number of consumed EC, EWC and OO were performed for all animals tested, for the phylum Arthropoda and for the phylum Echinodermata. Consumption of EC among phyla was also tested using a chi-square test. Tests among species and of the consumption of EWC and of OO for arthropods and for echinoderms were not possible to perform due to small sample size. Chi-square analyses were performed using JMP statistical software.

3.3.1. **Multivariate analysis** (Fig. 2A)

Multivariate Correspondence Analyses (MCA) were performed to explore which traits or combination of traits, if any, could explain the experimental results obtained in laboratory. An MCA is a multivariate statistical analysis equivalent to a Principal Component Analysis (PCA) but for categorical variables. However, contrary to PCA,

the variance explained by each MCA axis has no significant meaning and should not be taken into account (Telechea et al., 2009).

The experimental results were grouped into a variable named ‘Experimental outcome’ and coded as EC, EWC and OO (which corresponded to eating the food option EC, EWC or OO offered) and as No EC, No EWC and No OO (which corresponded to not eating the food option offered). Only the results of species tested with all 3 food options of the experiment were used for this analysis. Feeding biology of the tested species was characterised using 4 biological categories: trophic group (TG), feeding strategy (FS), capture strategy (CS) and capture tools (CT). Each category had several levels to which a species was assigned. Levels corresponded to functional attributes that grouped species within each biological category. For example, the category TG was composed by the functional groups; carnivore, detritivore and omnivore. A definition of the levels of each category, as used in this study, is provided in Table A4. Classification of the species within the categories was done based on the literature. When a species was known to have several traits or strategies of the same biological category, the predominant one was chosen.

The biological categories, the species and the experimental outcome were used to construct a categorical matrix. Species and experimental outcome were analysed with one or 2 of the biological categories at a time. This resulted in a total of 10 different combinations of variables which were considered as hypotheses (Fig. 2B). For example; hypothesis 1 (h1) assumed that the trophic group was the biological category that better explained the experimental results and the matrix for this MCA analysis was constructed using the variables; species, experimental outcome and trophic group. An MCA was performed for each hypothesis. Rejection of a hypothesis was determined visually based on the resulting MCA graph. No analyses were performed using more than 2 biological categories because when we performed MCA analyses using 2 biological categories, which in our study corresponded to the hypotheses 5 through 10 (h5 – h10) (Fig. Y2), the levels of the variable ‘experimental outcome’ were plotted with little association to the variable ‘species’ and to the other variables used. Thus, we considered this to be a sign that, in these analyses, the weight of the two biological categories was higher than the results of the experiment and therefore were not being reflected. For this reason, we considered that MCA analyses carried out with three or

more of the biological variables would lead to this same situation. MCA analyses were performed using the R package FactoMineR (Lê et al., 2008).

4. RESULTS

4.1. Feeding response of potential predators

Consumption of the three types of food offered (EC, ECW and OO) was significantly different among the potential predators tested (χ^2 = 62.13, p <0.0001). Of the 22 potential predator species, 13 ate none of the options presented in the feeding experiments and 9 ate two or more options (Table 1).

Table 1. Number of individual potential predators used and percentage of individuals that consumed eggs, eggs without capsule and oocytes per species. (EC, eggs with capsule; EWC, eggs without capsule; n, number of animals tested; N/A, not applicable; OO, oocytes). *Species with insufficient individuals to perform complete experiments.

	EC		EWC		OO	
	n	%	n	%	n	%
Calliactis parasitica	2	0	2	50	2	100
Lineus lacteus	2	0	2	0	1	0
*Hexaplex trunculus**	1	0	0	N/A	0	N/A
*Stramonita haemastoma**	0	N/A	1	0	0	N/A
*Semicassis granulata undulata**	1	0	0	N/A	0	N/A
*Octopus vulgaris**	1	0	0	N/A	0	N/A
Nephtyid	3	0	3	0	3	0
Nereid 1	4	0	4	0	4	0
Nereid 2	4	0	4	0	4	0
Cancer pagurus	2	100	2	50	2	100
Carcinus maenas	5	0	5	100	5	100
Dardanus arrosor	4	75	4	100	4	100
Nephrops norvegicus	3	0	3	66,7	3	100
Parthenopoides massena	1	0	1	0	1	0
Upogebia sp.	4	0	4	0	4	0
Cidaris sp.	3	33, 3	3	66,7	3	66,7
Echinus melo	4	100	4	100	4	100
Paracentrotus lividus	2	100	1	0	1	100
Astropecten aranciacus	1	100	1	0	1	100
*Coscinasterias tenuispina**	1	0	0	N/A	0	N/A
Echinaster sepositus	2	0	2	0	2	0
*Peltaster placenta**	1	0	0	N/A	0	N/A

The species with only negative outcomes were the nemertean *L. lacteus* (*n* = 12), the polychaetes Nephtyid (*n* = 9), Nereid 1 (*n* = 12) and Nereid 2 (*n* = 12), the arthropods *Parthenopoides massena* (*n* = 3) and *Upogebia* sp. (*n* = 12) and the echinoderms *Coscinasterias tenuispina* (*n* = 1), *Echinaster sepositus* (*n* = 6) and *Peltaster placenta* (*n* = 1) (Table 1). None of the molluscan species tested consumed EC, which included the species *Hexaplex trunculus* (*n* = 1), *Stramonita haemastoma* (*n* = 1), *Semicassis granulata undulata* (*n* = 1) and *O. vulgaris* (*n* = 1) (Table 1). Nevertheless, the *H. trunculus* and *S. granulata undulata* species showed predation on EC when the exposition time was longer (11 days in total). The experiments with positive predatory outcomes belonged to the anemone *C. parasitica* (*n* = 6) and the remaining 6 arthropod (*n* = 57) and 7 echinoderm species (*n* = 36) (Table 1; Fig. A5). In these experiments, all species consumed OO and both the arthropods (χ^2 = 69.28, p <0.0001) and echinoderms (χ^2 = 11.20, p <0.0001) showed significant differences for consuming the 3 food types offered. Predation on EC differed significantly between phyla (χ^2 = 37.04, p <0.0001).

The sea urchin *Echinus melo* (*n* = 12) was the only species for which all individuals consumed both EC (Fig. A6F) and EWC. The crab *Cancer pagurus* (*n* = 6), the hermit crab *D. arrosor* (*n* = 12) (Fig. A6B) and the sea urchin *Cidaris* sp. (*n* = 9) also consumed both treatments but showed more variability among individuals. For instance, only 75% of *D. arrosor* and 33% of *Cidaris* sp. individuals consumed EC. EWC were consumed by 67% of *Cidaris* sp. and by 50% of *C. pagurus*. The species *Paracentrotus lividus* (*n* = 4) and *A. aranciacus* (*n* = 3) (Fig. A6E) also consumed EC, but did not eat EWC. On the other hand, EWC were also consumed by *C. parasitica*, the crab *Carcinus maenas* (*n* = 15) and the lobster *Nephrops norvegicus* (*n* = 9). Whereas all *C. maenas* individuals consumed EWC, only 50% and 67% of *C. parasitica* and *N. norvegicus* did so, respectively. These species did not consume EC.

4.2. Behavioural observations of potential predators

Although *C. parasitica* did not eat EC in the experiments, the individuals seemed to explore the EC before eventually rejecting it. On the other hand, the individuals of this species that ate EWC and OO started swallowing the item shortly after being offered. The *O. vulgaris* and *P. placenta* individuals both appeared to briefly explore the EC when offered, but did not seem further interested during the rest of the experiment. The species *C. maenas* and *N. norvegicus* were observed attempting to prey on EC but failed

to rupture the capsule, which showed clear marks on its surface at the end of the experiments. Some individuals of *N. norvegicus* also appeared to show some struggle to manipulate and consume EWC during their first attempts because EWC slipped from the grasp of chelipeds and due to the movement of these appendages the EWC might bounce and swing in the water. Sea urchins generally consumed all 5 EC offered in the experiment. However when this was not the case, the EC not consumed also showed marks on the external surface of the egg capsule. *A. aranciacus* was observed ingesting an EC (Fig. A6E) by swallowing it whole and regurgitated a cuttlebone (after 18 days) (Fig. A6D) and shells (Fig. A6C), the latter surely ingested before the experiment.

4.3. Capture tools of potential predators

MCA analyses for all hypotheses showed that the biological trait that seemed to better explain the results obtained in the laboratory experiment was the one performed with the variable 'capture tools' (h4) (Fig. 3).

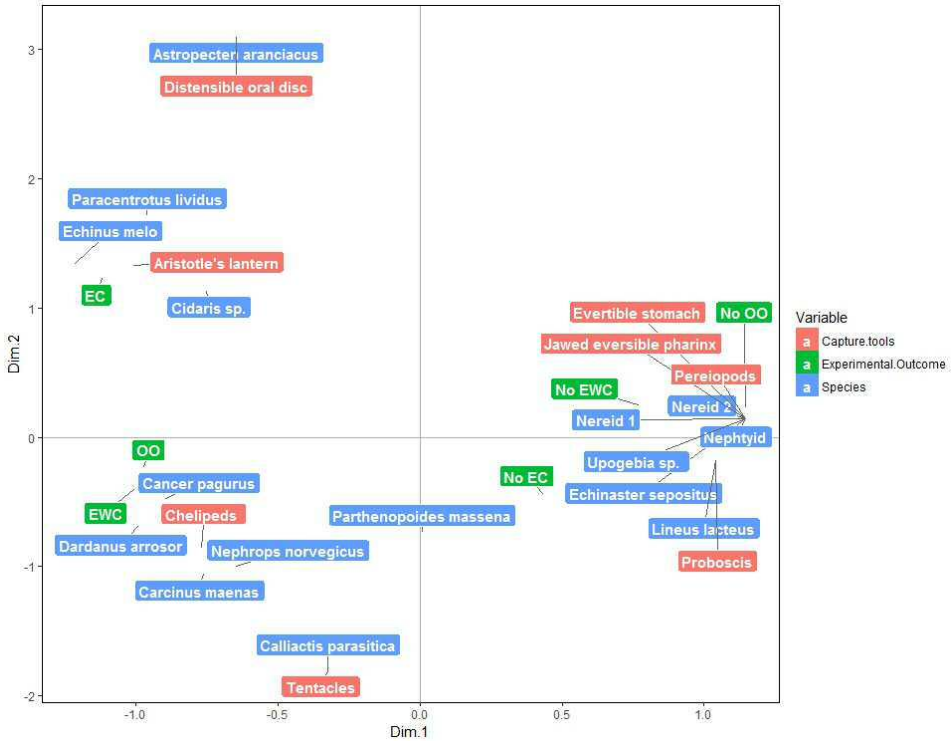

Fig. 3. Multivariate Correspondence Analysis of hypothesis 4; testing the laboratory results (represented by the variable Experimental outcome) per species, with the biological category Capture tools. (EC, eggs with capsule consumed; EWC, eggs without capsule consumed; OO, oocytes consumed; No EC, eggs with capsule not consumed; No EWC, eggs without capsule not consumed, No OO, oocytes not consumed).

MCA analyses performed with the variables for the other hypotheses did not group species taking into account the laboratory experiment and were rejected (Fig. A7). In the graph of the MCA (Fig. 3) performed with the variable 'capture tools', two main groups of species can be identified along the first axis. One group, located on the right part of the diagram, is characterised by not eating any of the food options of the experiment and the second group, located on the left part, is characterised by eating at least some of the food options. Within this group along the second axis, the species with chelipeds appear to be more associated with eating EWC and OO, whereas the species with an Aristotle's lantern or a distensible oral disc seem more related with eating EC.

5. DISCUSSION

Although predation might be an important threat for many benthic marine egg masses (Dumont et al. 2008), no major predation pressure has been reported for the relatively large eggs of the European cuttlefish *S. officinalis* (Guerra 2006). In this study, an experimental approach was used to expressly investigate this and results have showed that predation on egg masses of *S. officinalis* by benthic marine invertebrates may be possible. Mainly only crabs, sea urchins and a starfish (*A. aranciacus*) fed on *S. officinalis* eggs under our experimental conditions.

5.1. The feeding biology of non-predators of *Sepia officinalis* eggs

In our experiments we found that a diverse group of the tested species did not feed nor show interest for any of the food offered (EC, EWC or OO). This seems to indicate that this type of food material was not attractive to them. However, egg masses provide a rich source of nutrition and in particular, *Sepia* eggs are very rich in yolk, which represents approximately 13% of the total egg weight and, in essence, is a storage of energy rich in proteins, carbohydrates and lipids (Matozzo et al. 2015, Sykes et al. 2009). On the other hand, although this type of food material could have been edible for them, they might not have been able to detect it as food. Many animals have evolved sensory systems that will only recognize and locate food in the presence of certain substances or cues (Brusca & Brusca 2003). Therefore, to properly investigate why this group of species did not eat, detailed information of the feeding biology for species level would be needed. Interestingly, the majority of these species were either carnivorous or omnivorous, which are thought to include many kinds of eggs in their diets (Orians & Janzen 1974). Take for example the nemertean *L. lacteus*. Although it

belongs to a macrophagous genus that captures its prey by rapidly everting the proboscis and secreting substances that paralyse it (Thiel & Reise 1993), there are no previous studies on its feeding ecology. Yet, its related nemertean *Riseriellus occultus* is known to feed on gastropods and bivalves (Beckers et al., 2015). Thus, the behaviour of *L. lacteus* in these experiments would not be consistent with the probable natural feeding behaviour of the species. Whereas the negatives results obtained with *Upogebia* sp. were in accordance with its known feeding biology; a detritivorous animal that generates water currents and feeds on suspended material (Dworschak 1987).

5.2. The ambiguous case of the molluscan species

In our experiments, *O. vulgaris* and the gastropods did not show predation on EC (or EWC for the gastropod *S. haemastoma*). However, it should be noted that these results pertain only to one observation and so should be regarded with caution and considered primarily as case studies to further explore. This is especially evident for the species *H. trunculus* and *S. granulata undulata* which yielded negative results using our experimental design but that could produce a different outcome for experiments with either a longer starvation period or longer exposure time to eggs, since these species showed predation when the exposition time to EC was longer. Besides, *H. trunculus* is believed to be an opportunistic predator that may also scavenge, that thrives in ultra-oligotrophic marine waters off the coast of Israel, where food is limited (Peharda & Morton 2006) and in aquarium can survive up to months without feeding (Sawyer et al. 2009). Thus, a longer experiment might be more suitable to investigate feeding traits of species like this one.

5.3. The feeding biology of potential predators of *Sepia officinalis* eggs

Traditionally, sea urchins are considered predominantly herbivores but they can consume a wide variety of animal protein and for many, an omnivorous or unambiguously carnivorous diet has been reported (Crook & Davoren 2016, Jangoux & Lawrence 1982). Even though *P. lividus* appears to have a reduced ability to assimilate animal material when compared with other sea urchins like *Arbacia lixula* (Wangensteen et al. 2011), it showed predation on EC in the laboratory experiments. This was also the case for the other two echinoids tested; *Echinus melo* and *Cidaris* sp. Thus, the egg capsule did not appear to oppose as much resistance for echinoids. To feed, this group depends mainly on the Aristotle's lantern, a complex masticatory

structure located inside the mouth that bears several calcareous protractible teeth (Brusca & Brusca 2003). This tough structure is also capable of a variety of movements of the teeth, including protraction, retraction and grasping and many species are able to protrude the teeth at different angles (Brusca & Brusca 2003). Feeding on other marine benthic egg masses by sea urchins has been previously reported for *Strongylocentrotus droebachiensis* who is thought to be an important predator of egg masses of the whelk *Buccinum undatum* and an important source of mortality of other gastropod embryos (Dumont et al. 2008). Therefore, *S. officinalis* eggs could be part of the diet of littoral sea urchins with more carnivorous or omnivorous feeding habits. Aside from sea urchins, EC were consumed by predators with either carnivorous or omnivorous diets; the starfish *A. aranciacus*, known to be a voracious predator with a carnivorous diet (Baeta & Ramón 2013) and the two crabs, *Cancer pagurus* and *Dardanus arrosor*. Both these crabs are active predators although hermit crabs are generally omnivorous and display a range of feeding strategies (Hazzlet 1981). For instance, the congeneric of *D. arrosor*, *D. setifer* is known to be highly mobile and have an opportunistic predatory and scavenging behaviour, using its large chelae to grasp and break up its food or prey (Kunze & Anderson 1979). On the other hand, *C. pagurus* is considered a more sluggish predator that predominantly ambushes epifaunal prey without distancing itself much from its refuge or its proximities (Lawton 1989). Another relevant feature is the fact that *C. pagurus* also exhibits a high degree of specialization on hard-shelled prey and possesses claws more powerful than omnivorous crabs (Yamada & Boulding 1998). In contrast, *Carcinus maenas*, a very active and voracious predator but with an omnivorous diet (Chaves et al. 2010, Ropes 1968), has relatively smaller, thinner chelae with less crushing strength (Moody & Steneck 1993). Interestingly, this is one of the species that consumed EWC but were not able to prey on EC. The other decapod that consumed EWC and failed to prey on EC was *N. norvegicus*, also a predator with scavenging activity and a generalist diet (Cristo & Cartes 1998). This species captures its prey or food material grasping it with the chelipeds and walking legs (Bell et al. 2013) which may not have the strength and dexterity to rupture the egg capsule but that would allow them to feed on eggs of *S. officinalis* if these are protected only by the chorion membrane. The other species that consumed EWC and did not feed on EC was the anemone *C. parasitica*, which generally lives attached to gastropod shells inhabited by hermit crabs (Ross & Sutton 1961). Although generally a suspension feeder, it is characterized for having a very omnivorous generalist diet because it also preys on a

wide spectrum of organisms living in the sediment (Chintiroglou & Koukouras 1991). In particular, this species is known to twist its body so that the entire expanded oral disc becomes almost parallel to the substratum allowing its tentacles to swipe the substratum, but it also eats food particles of large prey captured and tore into pieces by the hermit crab that come into contact with its tentacles (Chintiroglou & Koukouras 1991). On the other hand, predatory behaviour by *C. parasitica,* and other sea anemones, has also been documented under determined environmental conditions (Riedel et al. 2008).

Moreover, the results of the echinoderms *A. aranciacus* and *P. lividus* are noticeable: although they consumed EC, they did not consume EWC in the experimental conditions. These results, however, seem more likely to be an anomaly or due to individual variability rather than indicate that the chorion membrane was acting as an effective protective barrier since other individuals of these species were able to break the barrier posed by the egg capsule when offered EC. On the other hand, all species that ingested EC and/or EWC also consumed OO, suggesting that OO had an insufficient physical barrier to protect the food material from predation.

5.4. Egg capsule and chorion membrane protective features

Performing MCA analyses with different combinations of the biological variables allowed us to identify the method of food capture (i.e. capture tools) as the biological trait that appeared to best explain the results of the feeding laboratory experiments. Given that whether or not eggs of *S. officinalis* were eaten appeared to be determined by a mechanical factor, this finding seems to highlight the importance of having the tools capable of surpassing the resistance of a mechanical barrier.

The *S. officinalis* embryo develops surrounded by different layers that shield it from the surrounding environment (Boletzky 1986, Lacoue-Labarthe et al. 2016). The most important barrier against environmental damages is the egg capsule (Boletzky 1998). Its resistance properties are evidenced for several of the species tested here as potential predators, especially for those, like *C. maenas,* that showed an intention to prey on eggs but did not surpass the egg capsule. Nonetheless, these protective properties were not sufficient for species with powerful capture tools, like *C. pagurus* or the echinoids, which successfully ruptured the egg capsule. On the other hand, egg capsules also undergo changes in close relation to the metabolism of the embryo (Boletzky 1998). For

instance, towards the end of the embryological development the egg capsule becomes thinner and more transparent (Wolf et al. 1985, Cornet et al. 2015). Consequently, predation on eggs at different stages of development might also differ. However, this aspect was not evaluated in the present study. Furthermore, even if an animal fails to prey on *S. officinalis* eggs, damages to the capsule may be made in the process of inspection that might be harmful to the viability of the embryo. Even a small hole may constitute a point of entry to a variety of microfauna and hinder the survival of the embryo. Also, it might make eggs more likely to detach from the egg masses and from its substrate leading to, for example, scenarios of reduced oxygen diffusion due to the egg resting and rolling over the sediment and/or surface abrasion of the capsule, which would diminish embryo survival. Thus, manipulation of these eggs by benthic animals, even without predation, might also contribute to egg mortality.

The chorion membrane is the chronological primary egg cover and the primary egg protection (Boletzky 1986, Boletzky 2010). However, by itself this structure is insufficient for effective protection of the embryo from the outside environment (Boletzky 2010). This is supported in our laboratory results since species that appeared to be attracted to this type of food were able to feed on eggs only protected by the expanded chorion membrane (EWC). As far as we know, this is a membrane that also acts as a passive barrier for many molecules (Bonnaud et al. 2013, Lacoue-Labarthe et al. 2016). Thus, it could also govern the diffusion of attractive odours that might be detected by other putative predators. Moreover, it is a structure that hinders the manipulation of the egg, evidenced by the scenario observed for individuals of *N. norvegicus* where the EWC had a tendency to swing and bounce with the movement of the chelipeds.

The structural protection of *S. officinalis* egg masses might be coupled with chemical defence mechanisms, which should not be overlooked either. This potential chemical protection might be provided by ink, living bacteria inside the egg capsule and antifouling properties and antimicrobial properties of egg capsule proteins (Cornet et al. 2015). In particular, ink has been suggested to function as a chemical deterrent (Derby 2014) and it might provide a repulsive smell to ward off predators when imbedded in the egg capsule (Cornet et al. 2015).

6. CONCLUSION

This study has showed that predation on egg masses of the European cuttlefish *Sepia officinalis* by benthic marine invertebrates may be possible. Under laboratory conditions, 22 different invertebrate species of 6 different phyla and with diverse feeding habits were tested as potential predators of *S. officinalis* eggs, revealing that these were preyed on mainly by crabs, sea urchins and a starfish (*Astropecten aranciacus*). The laboratory results are not enough to support the concept that species that showed predation on eggs with capsule (EC) should be considered a predator of *S. officinalis* eggs, even more so if we bear in mind that in the field other prey will be available for these predators. Nonetheless, it can be argued that cuttlefish eggs could be part of their diet; at least for certain periods of time since changes in prey availability or environmental conditions may induce shifts in predator diets (Pickering & Quijón 2011). Regarding the egg capsule of *S. officinalis*, evidence of its mechanical protective functions is presented. The ability of the chorion membrane to act as a physical barrier and hinder the manipulation by invertebrate animals of a *S. officinalis* egg protected only by this membrane was also explored. Moreover, the results obtained suggest that egg masses of *S. officinalis* may be vulnerable to some benthic invertebrate predators and that eggs rely on passive structural characteristics to avoid predation, though chemical properties and putative deterrents also have to be considered and should be investigated further.

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8. APPENDIX

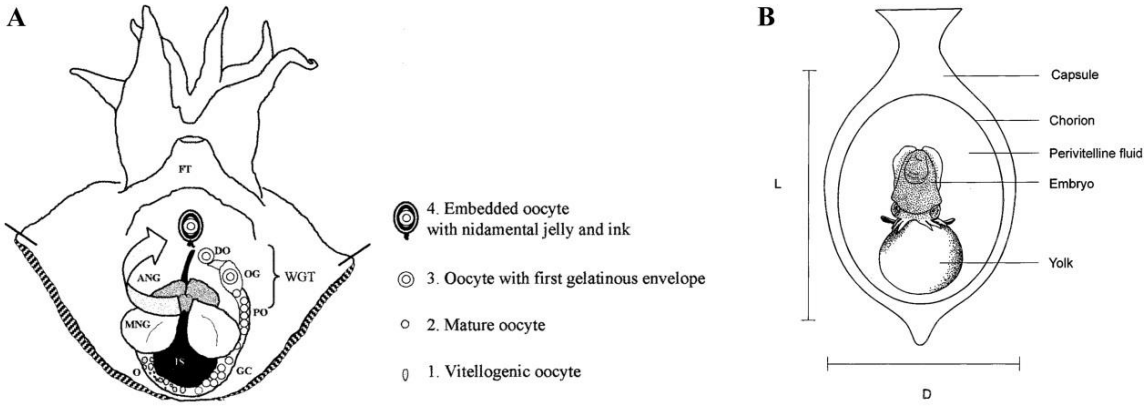


Fig. A1. Schematic drawings of (A) the internal structures and steps of ovulation and enveloping of eggs in a mature *Sepia officinalis* female from ventral view (taken from Zatylny et al. 2000) and (B) of a *Sepia* egg (taken from Cronin & Seymour 2000). (ANG, accessory nidamental glands; D diameter; DO, distal oviduct; FT, funnel tube; GC, genital coelome; IS, ink sac; L external egg length; MNG, main nidamental glands; O, ovary; OG, oviducal gland; PO, proximal oviduct).

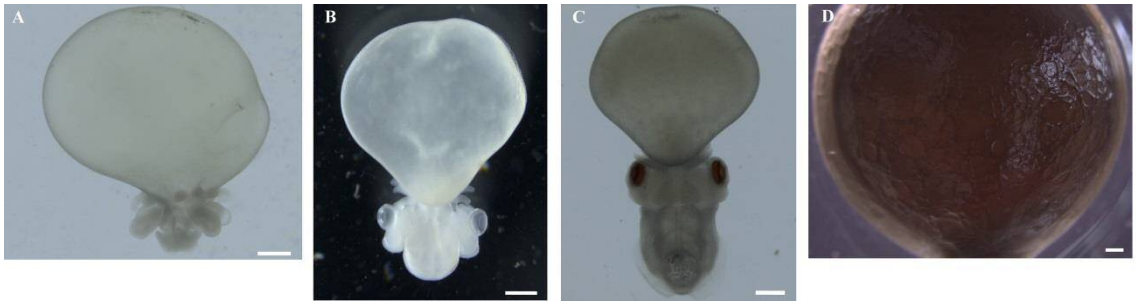


Fig. A2. *Sepia officinalis.* Embryos at embryological stages (A) 21, (B) 23 and (C) 28 (after Boletzky et al., 2016). (D) Surface of the expanded egg capsule of an egg approaching the end of embryological development. Scale bars: 1mm.

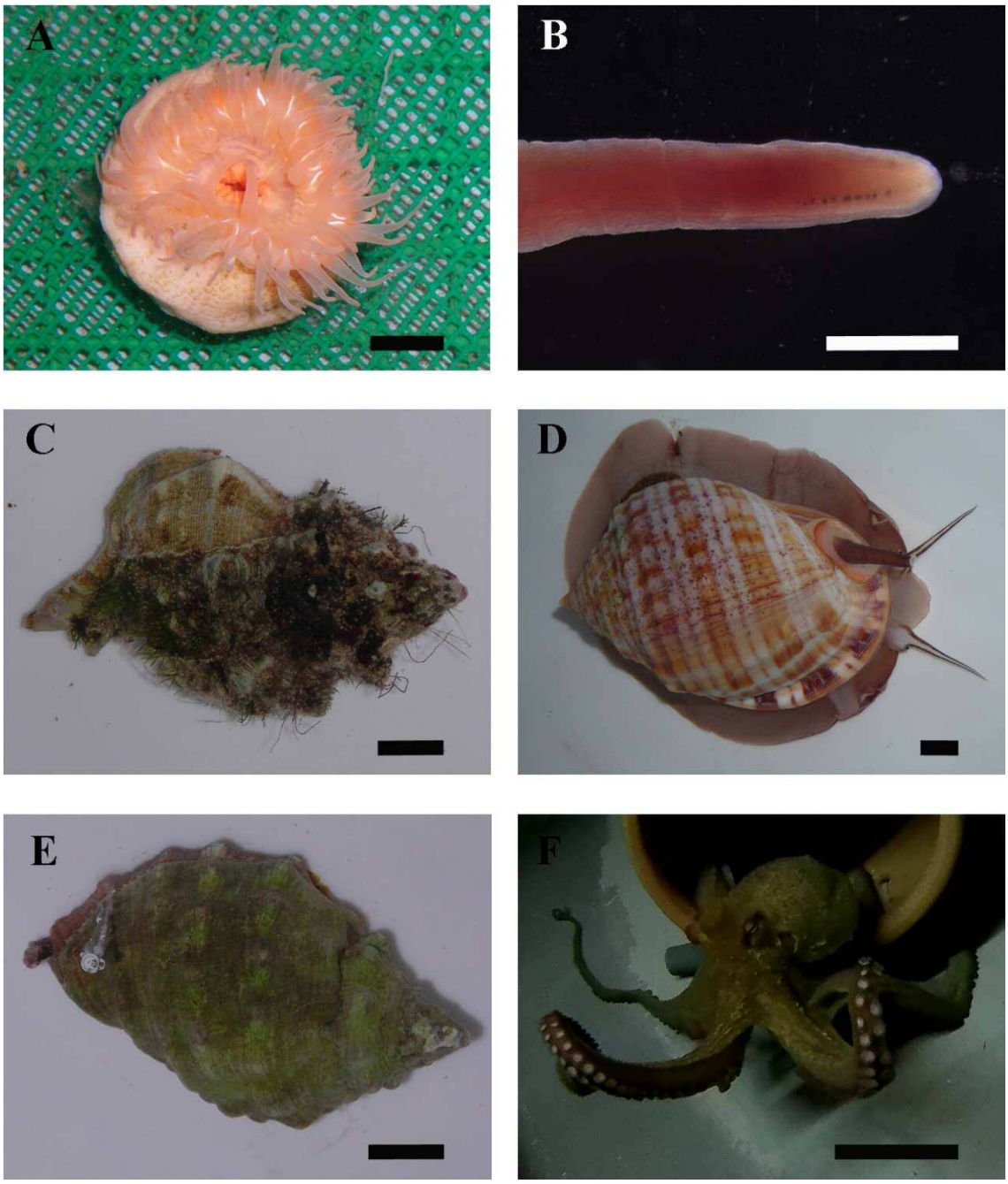


Fig. A3. Species tested as potential predators of *Sepia officinalis* eggs. (A) *Calliactis parasitica*. (B) *Lineus lacteus*. (C) *Hexaplex trunculus*. (D) *Semicassis granulata undulata*. (E) *Stramonita haemastoma.* (F) *Octopus vulgaris.* (G) Nephtyid. (H) Nereid 1. (I) Nereid 2. (J) *Cancer pagurus*. (K) *Carcinus maenas*. (L) *Dardanus arrosor*. (M) *Nephrops norvegicus*. (N) *Parthenopoides massena*. (O) *Upogebia* sp. (P) *Cidaris* sp. (Q) *Echinus melo*. (R) *Paracentrotus lividus.* (S) *Astropecten aranciacus*. (T) *Coscinasterias tenuispina*. (U) *Echinaster sepositus*. Scale bars: 1 cm, except B and F: 1mm and 10 cm respectively.

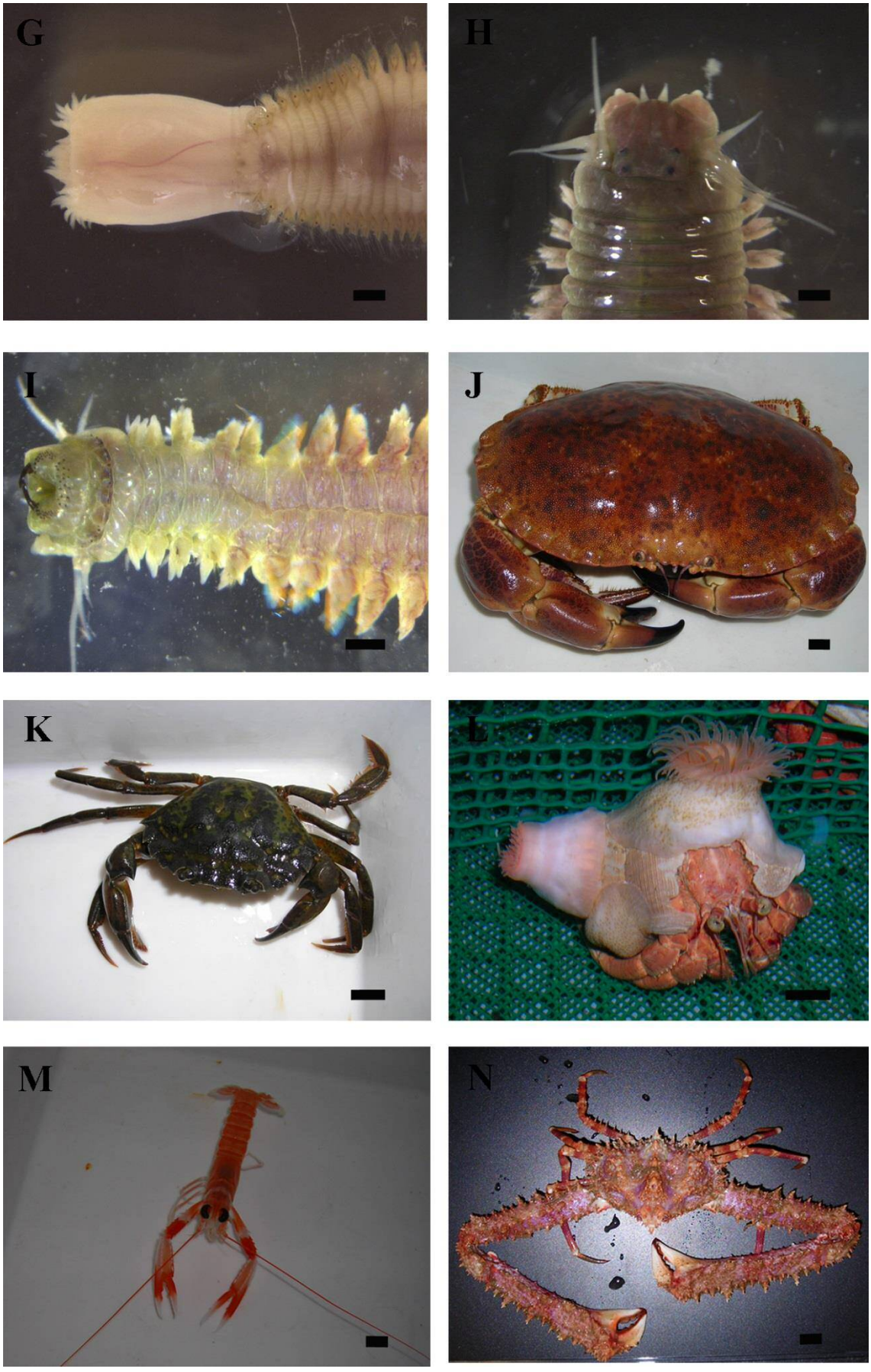
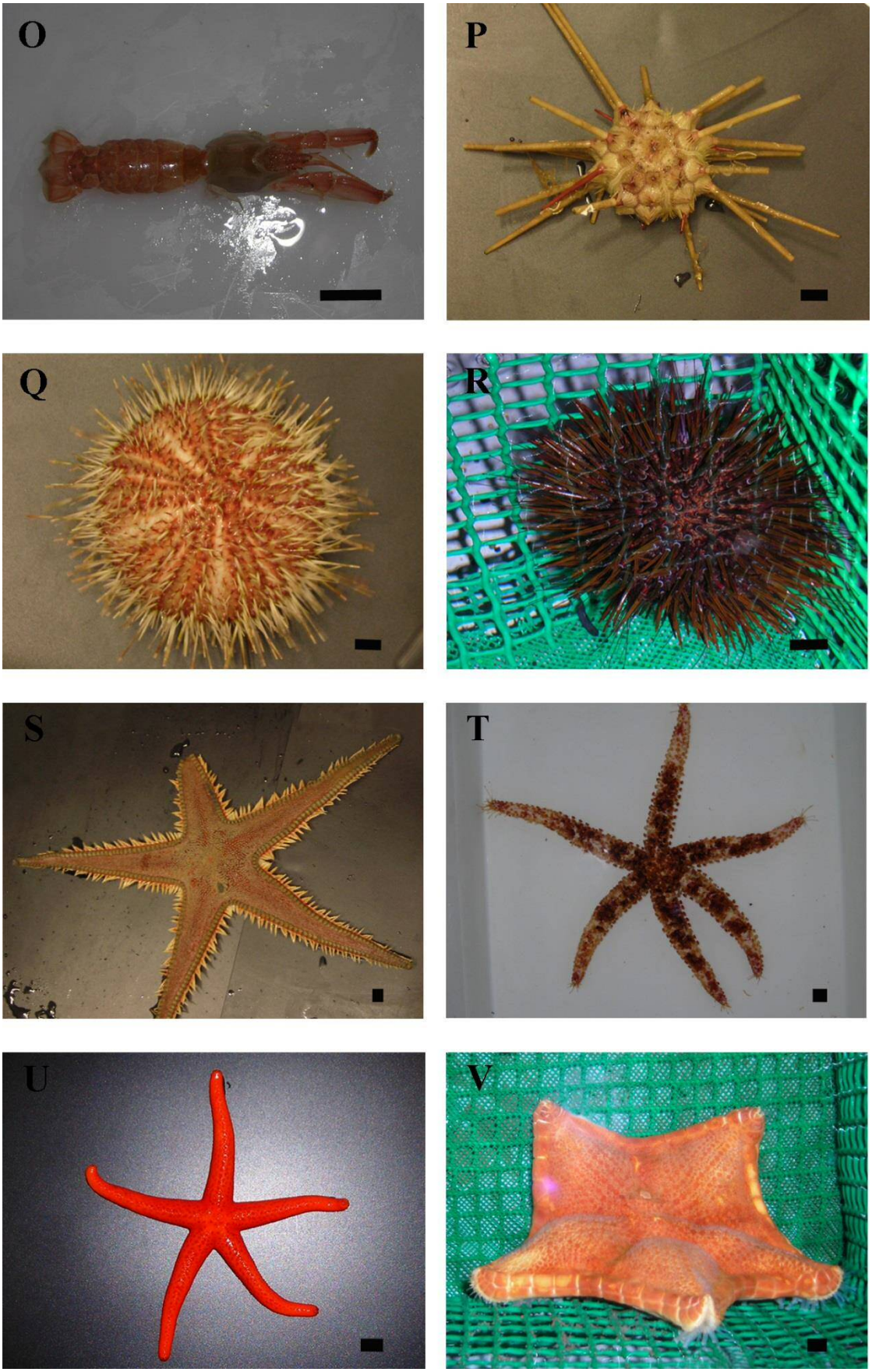


Fig. A3. Continued.



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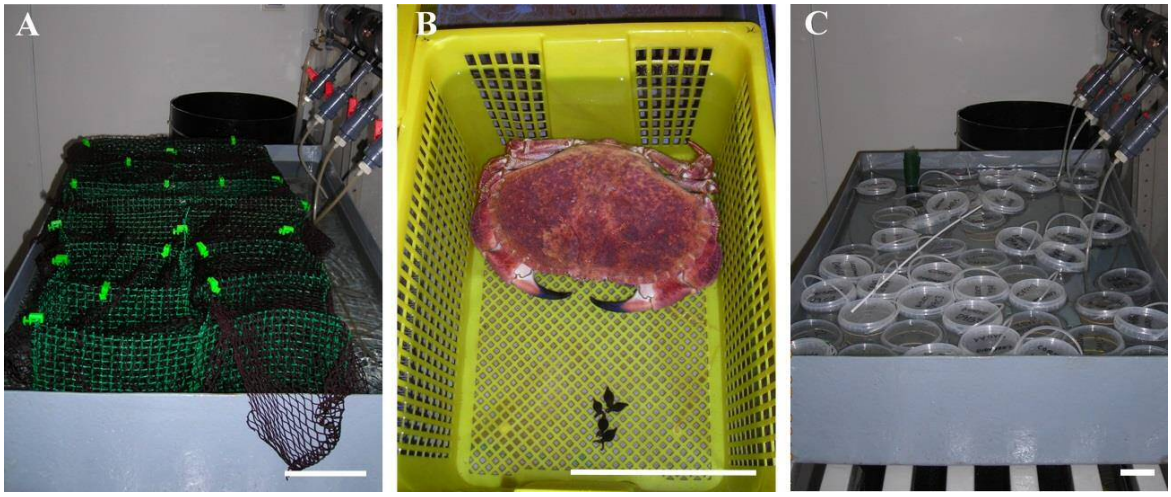

Fig. A4. Experimental set-up using (A) chambered cages of green wire mesh, (B) baskets and (C) plastic containers. Scale bars: 10 cm.

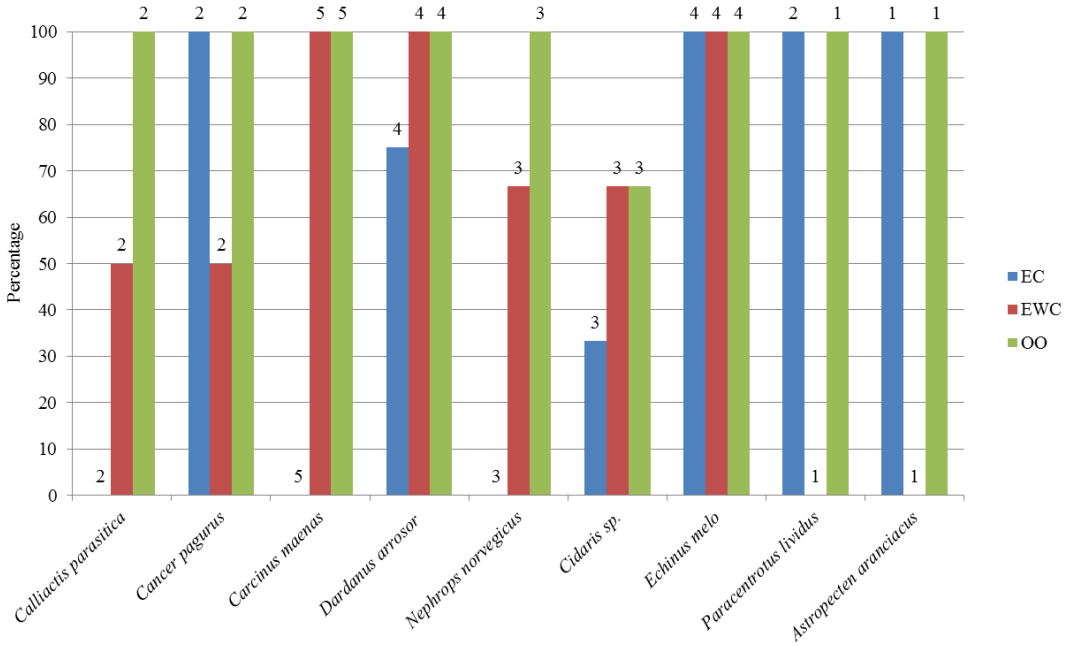

Fig. A5. Percentage of cnidarian, arthropod and echinoderm individuals that consumed EC, EWC or OO per species. Numbers on top of column bars indicate number of individuals tested. (EC, eggs with capsule; EWC, eggs without capsule; OO, oocytes).

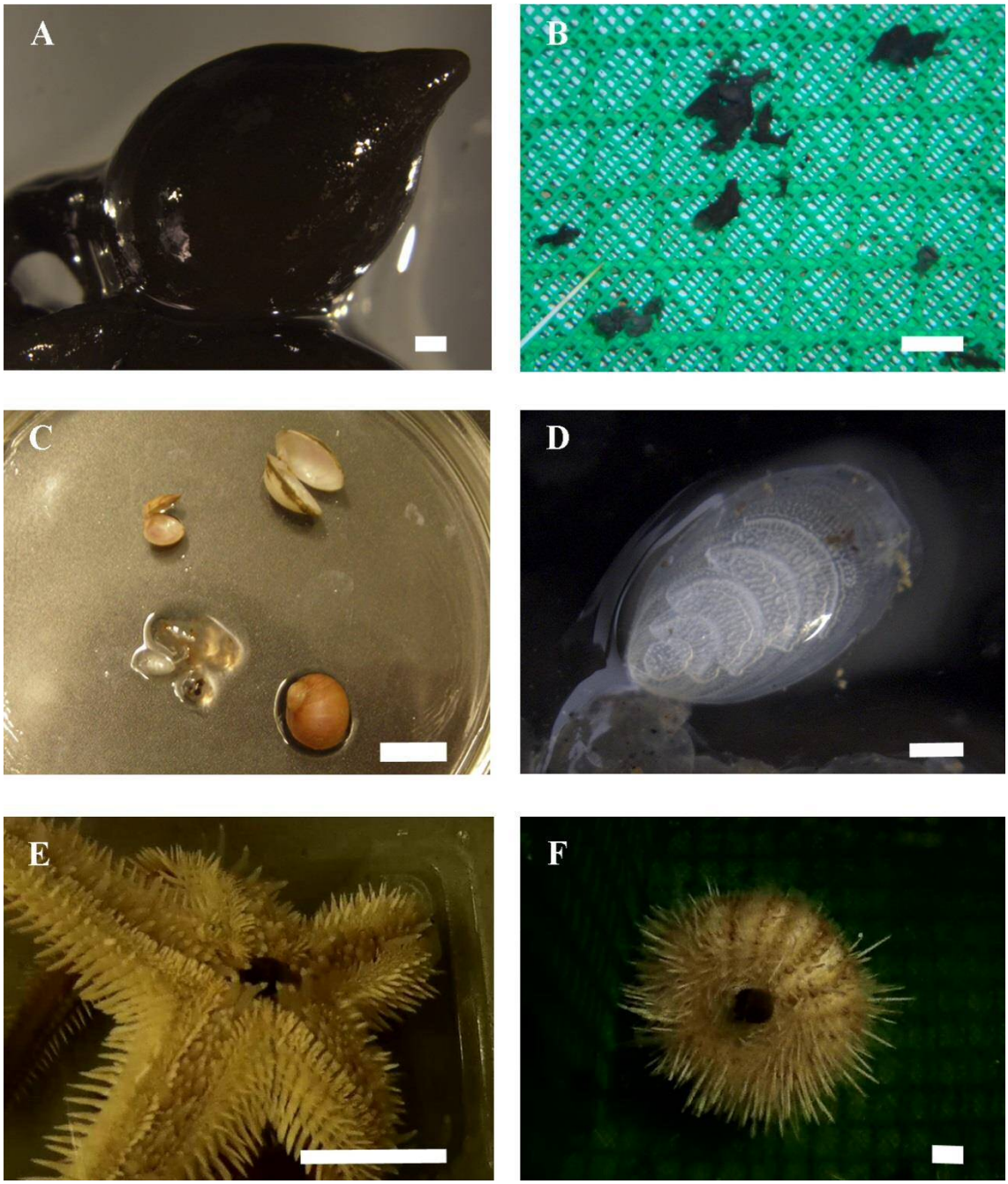

Fig. A6. Different stages of predation of *Sepia officinalis* eggs. (A) Egg with no marks of predation on the surface of the egg capsule. (B) Remainders of egg capsules after predation of eggs by *Dardanus arrosor*. (C) Regurgitated shells and cuttlebone by *Astropecten aranciacus*. (D) Detail of a regurgitated cuttlebone by *A. aranciacus*. (E) Individual of *A. aranciacus* eating an egg with capsule. (F) Individual of *Echinus melo* eating and egg with capsule. A and D scale bars: 1 mm. B, C and F scale bars: 1 cm. E scale bar: 10 cm.

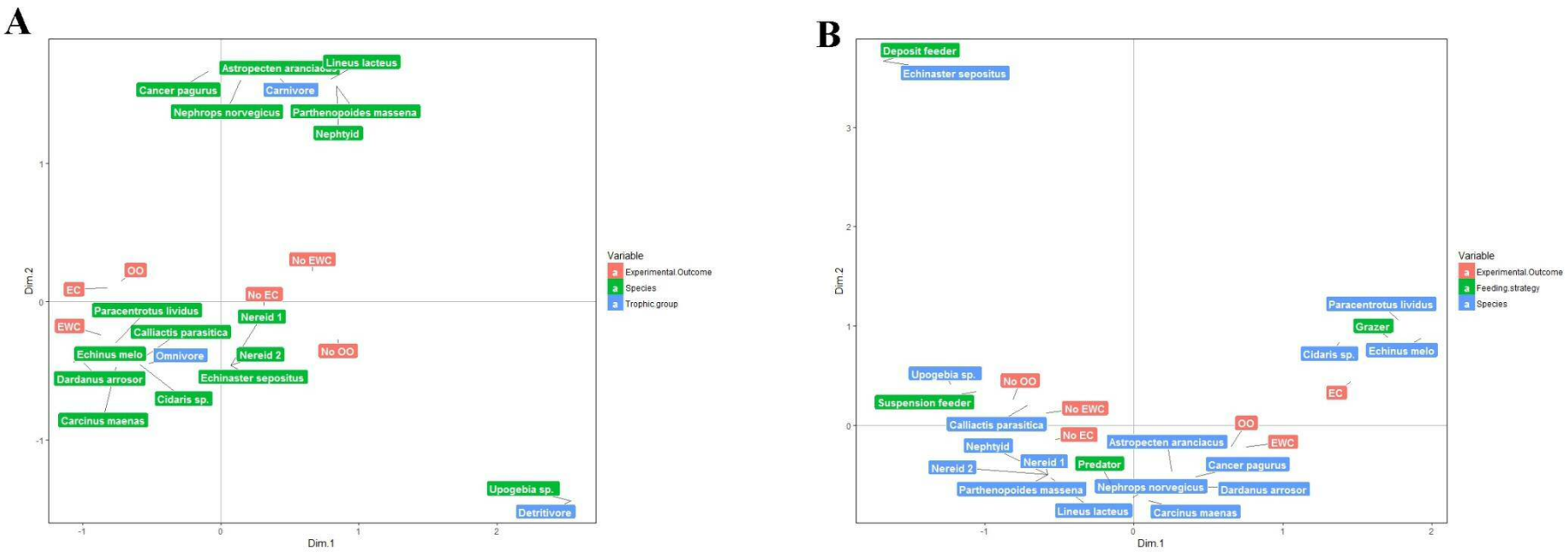

Fig. A7. Multiple Correspondence Analyses of (A) hypothesis 1, (B) hypothesis 2, (C) hypothesis (3), (D) hypothesis 5, (E) hypothesis 6, (F) hypothesis 7, (G) hypothesis 8, (H) hypothesis 9 and (I) hypothesis 10. (EC, eggs with capsule consumed; EWC, eggs without capsule consumed; OO, oocytes consumed; No EC, eggs with capsule not consumed; No EWC, eggs without capsule not consumed, No OO, oocytes not consumed).

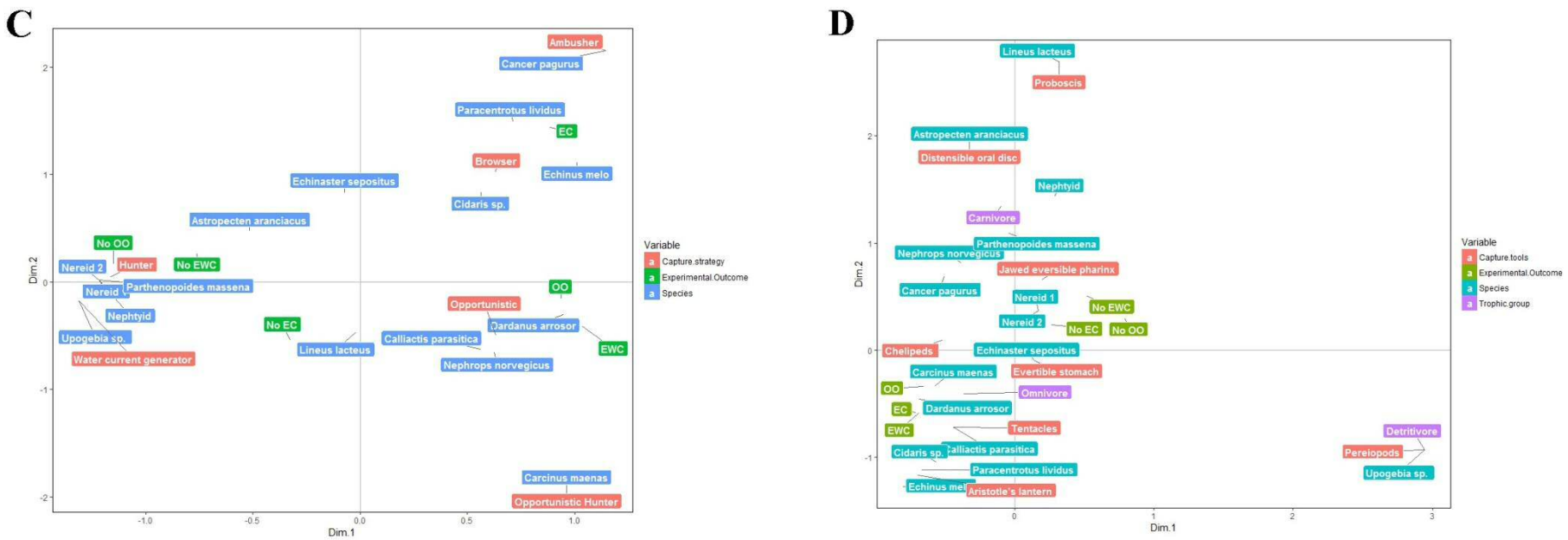

Fig. A7. Continued.

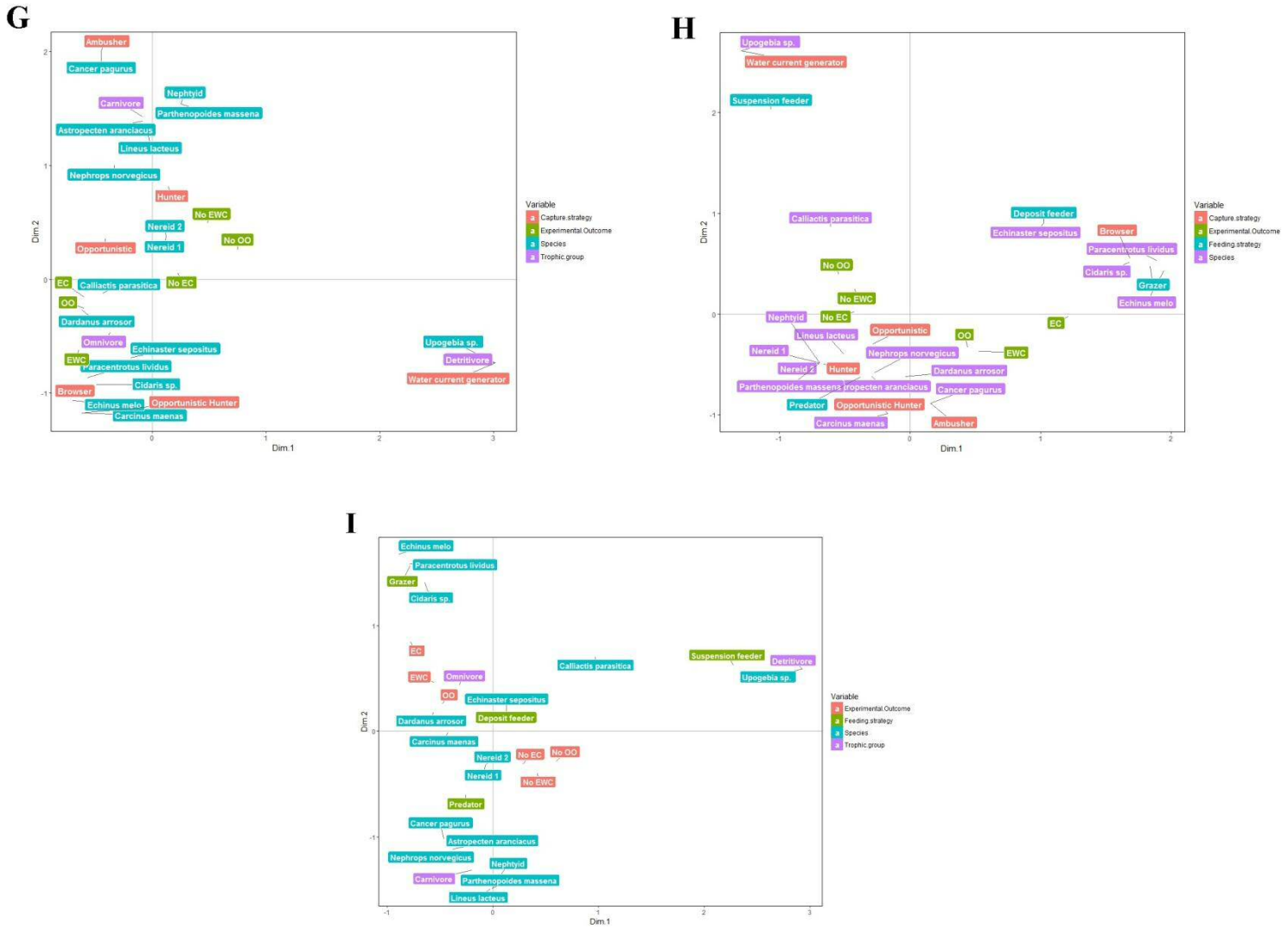

Fig. A7. Continued.

Table A1. Species tested as potential predators during the present study. All measurements are expressed as mean ± standard deviation. BS, body size; n, number of individuals tested; N/A, not applicable; WBW, wet body weight.

	Common name	n	BS (mm)	WBW (g)	Origin	Collection method
Phylum Cnidaria						
Class Anthozoa						
Family Hormathiidae						
Calliactis parasitica	Parasitic anemone	6	30.7 ± 8.3	15.0 ± 3.4	NW Mediterranean Sea	Trawler fisheries bycatch
Phylum Nemertea						
Class Anopla						
Family Lineidae						
Lineus lacteus		12	134.4 ± 31.3	0.12 ± 0.03	NE Atlantic Ocean	Direct sampling
Phylum Mollusca						
Class Gastropodoa						
Family Muricidae						
Hexaplex trunculus	Banded dye-murex	1	69.4	44.2	NW Mediterranean Sea	Aquarium of Barcelona
Stramonita haemastoma	Red-mouthed rock shell	1	55.1	27.5	NW Mediterranean Sea	Aquarium of Barcelona
Family Cassidae						
Semicassis granulata undulata	Mediterranean Bonnet	1	79.7	153.6	NW Mediterranean Sea	Aquarium of Barcelona

Table A1. Continued.

	Common name	n	BS (mm)	WBW (g)	Origin	Collection method
Class Cephalopoda						
Family Octopodidae						
Octopus vulgaris	Common octopus	1	144	1420	NW Mediterranean Sea	Artisanal pots fisheries
Phylum Annelida						
Class Polychaeta						
Family Nephtyidae						
Nephtyid		9	123.0 ± 41.4	2.3 ± 0.5	NE Atlantic Ocean	Bait market
Family Nereididae						
Nereid 1		12	210.5 ± 42.8	5.1 ± 0.9	NE Atlantic Ocean	Bait market
Nereid 2		12	60.7 ± 25.2	1.0 ± 0.3	Pacific Ocean	Bait market
Phylum Arthropoda						
Class Malacostraca						
Family Cancridae						
Cancer pagurus	Edible crab	6	109.5 ± 6.5	880.0 ± 169.2	NE Atlantic Ocean	Fish market
Family Carcinidae						
Carcinus maenas	Green crab	15	35.5 ± 5.8	24.5 ±7.9	NE Atlantic Ocean	Fish market
Family Diogenidae						
Dardanus arrosor	Striated hermit crab	12	10.5 ± 2.1	54.4 ± 13.0	NW Mediterranean Sea	Trawler fisheries bycatch

Table A1. Continued.

	Common name	n	BS (mm)	WBW (g)	Origin	Collection method
Family Nephropidae						
Nephrops norvegicus	Norway lobster	9	26.9 ± 3.2	9.3 ± 2.5	NW Mediterranean Sea	Trawler fisheries
Family Parthenopinae						
Parthenopoides massena		3	32.3 ± 5.0	32.3 ± 19.5	NW Mediterranean Sea	Trawler fisheries bycatch
Family Upogebiidae						
Upogebia sp.		12	11.6 ± 1.5	1.2 ± 0.5	W Mediterranean Sea	Bait market
Phylum Echinodermata						
Class Echinoidea						
Family Cidaridae						
Cidaris sp.		9	36.1 ± 9.8	50.4 ± 37.0	NW Mediterranean Sea	Trawler fisheries bycatch
Family Echinidae						
Echinus melo	Melon sea urchin	12	67.4 ± 7.7	147.0 ± 47.0	NW Mediterranean Sea	Trawler fisheries bycatch
Family Parechinidae						
Paracentrotus lividus	Purple sea urchin	4	57.2 ± 4.5	63.4 ± 5.8	NW Mediterranean Sea	Aquarium of Barcelona

Table A1. Continued.

	Common name	n	BS (mm)	WBW (g)	Origin	Collection method
Class Asteroidea						
Family Astropectinidae						
Astropecten aranciacus	Red comb star	3	273.3 ± 57.1	179.2 ± 89.6	NW Mediterranean Sea	Trawler fisheries bycatch
Family Asteriidae						
Coscinasterias tenuispina	Blue spiny starfish	1	215.0	58.1	NW Mediterranean Sea	Aquarium of Barcelona
Family Echinasteridae						
Echinaster sepositus	Red starfish	6	140.5 ± 20.9	30.2 ± 11.2	NW Mediterranean Sea	Trawler fisheries bycatch
Family Goniasteridae						
Peltaster placenta		1	182.0	N/A	NW Mediterranean Sea	Trawler fisheries bycatch

Table A2. Bathymetric distribution overlap of tested potential predator species and spawning bathymetric distribution of *Sepia officinalis* in the north-east Atlantic and/or Mediterranean Sea, and predominant feeding traits per species. Bathymetric distribution overlap is coded as 'total overlapping' when the spawning depth range of *S. officinalis* is totally covered; 'partial overlapping' when distribution coincides only with a portion of the spawning depth range; and 'bordering' when spawning maximum depth coincides with the minimum depth distribution of the tested species. ABO, accessory boring organ; N/A, not applicable.

	Bathymetric Distribution	**References**	**Trophic group**	**Feeding strategy**	**Capture strategy**	**Capture tools**	**References**
Calliactis parasitica	Total overlapping	Chintiroglou & Koukouras 1992	Omnivorous	Suspension feeder	Opportunistic	Tentacles	Chintiroglou & Koukouras 1991
Lineus lacteus	Total overlapping	Gibson 1995	Carnivorous	Predator	Opportunistic	Proboscis and mouth	McDermott & Roe 1985, Thiel & Kruse 2001
Hexaplex trunculus	Total overlapping	Chiavarini et al. 2003	Carnivorous	Predator	Opportunistic	Proboscis, radula & ABO	Morton et al. 2007
Semicassis granulata undulata	Total overlapping	Cordeiro et al. 2015	Carnivorous	Predator	Hunter	Proboscis, radula & ABO	Heller 2015
Stramonita haemastoma	Total overlapping	Cordeiro et al. 2016	Carnivorous	Predator	Hunter	Proboscis, radula & ABO	Watanabe & Young 2006
Octopus vulgaris	Total overlapping	Norman et al. 2016	Carnivorous	Predator	Hunter	Arms	Norman et al. 2016
Nephtyid*	N/A	N/A	Carnivorous	Predator	Hunter	Jawed eversible pharinx	Fauchald & Jumars 1979
Nereid 1*	N/A	N/A	Omnivorous	Predator	Hunter	Jawed eversible pharinx	Fauchald & Jumars 1979, Caron et al. 2004
Nereid 2*	N/A	N/A	Omnivorous	Predator	Hunter	Jawed eversible pharinx	Fauchald & Jumars 1979, Caron et al. 2004

Table A2. Continued.

	Bathymetric Distribution	References	Trophic group	Feeding strategy	Capture strategy	Capture tools	References
Cancer pagurus	Total overlapping	Harrison & Crespi 1999	Carnivorous	Predator	Ambusher	Chelipeds	Lawton & Hughes, 1985
Carcinus maenas	Total overlapping	Chaves et al. 2010	Omnivorous	Predator	Opportunistic hunter	Chelipeds	Ropes 1968, Chaves et al. 2010
Dardanus arrosor	Total overlapping	Abelló et al. 1988	Omnivorous	Predator	Opportunistic	Chelipeds	Caine 1975, Kunze & Anderson 1979
Nephrops norvegicus	Bordering	Sardà 1998	Carnivorous	Predator	Opportunistic	Chelipeds	Bell et al. 2013, Cristo & Cartes 1998
Parthenopoides massena	Partial overlapping	Abelló et al. 1988	Carnivorous	Predator	Hunter	Chelipeds	Mura & Orrù 1998
Upogebia sp.	Total overlapping	Dworschak 1987	Detritivorous	Suspension feeder	Water current generator	Pereiopods	Dworschak 1987
Cidaris sp.	Bordering	Terribile & Schembri 2013	Omnivorous	Grazer	Browser	Aristotle's lantern	Jangoux & Lawrence 1982
Echinus melo	Partial overlapping	Minin 2012	Omnivorous	Grazer	Browser	Aristotle's lantern	Jangoux & Lawrence 1982
Paracentrotus lividus	Total overlapping	Lawrence 2013	Omnivorous	Grazer	Browser	Aristotle's lantern	Jangoux & Lawrence 1982
Astropecten aranciacus	Total overlapping	Baeta et al. 2016	Carnivorous	Predator	Hunter	Distensible oral disc	Baeta & Ramón 2013, Güler & Lök 2015
Coscinasterias tenuispina	Total overlapping	Micael et al. 2012	Carnivorous	Predator	Opportunistic	Evertible stomach	Lawrence 2013

Table A2. Continued.

	Bathymetric Distribution	References	Trophic group	Feeding strategy	Capture strategy	Capture tools	References
Echinaster sepositus	Total overlapping	Villamor & Becerro 2010	Omnivorous	Deposit feeder	Browser	Evertible stomach	Ferguson 1969, Villamor & Becerro 2010
Peltaster placenta	Total overlapping	Encyclopedia of Life	Omnivorous	Suspension feeder	Opportunistic	Pedicellaria	Jangoux & Lawrence 1982

Table A3. Description of the body size measurements used for the potential predator species. *Measurements taken from live animals and could contain certain variability attributed to the continuous contraction and stretching of their body; ** Shield length calculated using a regression function, provided by Pere Abelló (unpublished data) and the length of the first left chela; †Total length measured from the tip of an arm to the tip of the opposite arm.

Species	Measurement
Calliactis parasitica	Pedal disc diameter
Lineus lacteus *	Total Length
Hexaplex trunculus	Shell length
Semicassis granulata undulata	Shell length
Stramonita haemastoma	Shell length
Octopus vulgaris	Dorsal mantle length
Nephtyid*	Total Length

Table A3. Continued.

Species	Measurement
Nereid 1*	Total Length
Nereid 2*	Total Length
Cancer pagurus	Carapace length
Carcinus maenas	Carapace length
*Dardanus arrosor***	Shield length
Nephrops norvegicus	Carapace length
Parthenopoides massena	Carapace length
Upogebia sp.	Carapace length
Cidaris sp.	Oral diameter
Echinus melo	Oral diameter
Paracentrotus lividus	Oral diameter
Astropecten aranciacus	Total length†
Coscinasterias tenuispina	Total length†
Echinaster sepositus	Total length†
Peltaster placenta	Total length†

Table A4. Definition of the levels of the biological categories Trophic Group, Feeding Strategy and Capture Tools, as used in this study to characterize the feeding biology of the species of potential predators.

Biological category	Levels	Description
Trophic Group	Carnivore	Diet composed solely of animal material.
	Detritivore	Diet composed of detritus.
	Omnivore	Diet composed of animal and vegetal material.
Feeding Strategy	Deposit feeder	Stirs and ingests sediments of soft-bottom habitats (i.e. of sand and mud)
	Grazer	Scrapes substrates and tears pieces of tissue, which are then chewed.
	Predator	Actively captures and kills on live animals to feed.
	Suspension feeder	Removes and feeds on suspended food particles from the surrounding environment (by some sort of capture, trapping or filtration mechanism)
Capture Strategy	Ambusher	Animal sits and waits for its prey to come within capture distance.
	Browser	Animal moves about the substratum picking through what they encounter.
	Hunter	Animal actively searches and pursues its prey.
	Opportunistic	Animal has a distinctly generalist diet and may switch feeding strategy temporarily to feed on food more readily available or its ability to feed depends solely on passively coming in contact with food material.
	Opportunistic hunter	Animal has a distinctly generalist diet but also actively searches for specific prey.
	Water current generator	Animal creates directed water currents to collect the suspended food particles.

8.1. Appendix references

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